REMARKS

Claims 15-30 and 60-76 are pending and rejected. Claim 15 is presently amended. Favorable reconsideration of the pending claims is respectfully requested in view of the following amendment and remarks.

At the outset, applicant noted that the examiner identified, and PAIR reflects Applicant's prior submission as being filed on March 28, 2003. This appears to be in error as Applicant timely filed the response to the prior Office Action on May 06, 2002. This erroneous recording of Applicant's filing of the response detrimentally affects the calculation for this application patent term adjustment (PTA). PTAs were created to compensate applicants for undue delay by the Patent Office which would affect the term on the patent such as instantly suffered by applicant here. It should be to no detriment to the Applicant that the Patent Office was unable to act on the Reply until Applicants had shown by Petition that the reply was timely filed. Accordingly, Applicants kindly request that the date of entry of the prior Reply be corrected in the record and the PAIR system to reflect the actual date of submission of this paper to the Patent Office of May 6, 2002.

Applicant also wishes to bring to the Examiner's attention that the claims pending in this application are a continuation of the prosecution of certain prior claims which went to the Appeal Board in the prior application 07/833,973. A copy of the Appeal Board Decision in the parent application is kindly provided herewith. The 07/833,973 application contained two separate groupings of claims considered not to stand and fall together. Of these groups, one was allowed and resulted in Patent 6,174,999.

Applicant appreciates the withdrawal of the prior double patenting rejection over the U.S. patents 5,760,200 and 6,174,999 and now present below further arguments for reconsideration of the newly applied double patenting rejections.

Amendment to the Claims:

Claim 15 is amended to state more clearly what applicants regard as their invention. In particular, clarification as to the role of the modifying agent is introduced in the claim with the terms "causes the formation of a second activated species" in place of the terms "causes the formation of a new active carbonyl groups." (sic) Applicants also introduce language with regard to the formation of "a first activated species" to provide proper context to the term "second."

Support for this amendment can be found in the specification at least as follows:

- on page 6, lines 10 to 17, for the "first activated polysaccharide" wherein it is described that " A polyanionic polysaccharide is said to be 'activated' ... when it is treated ... in a manner that renders the carboxyl groups on the polyanionic polysaccharide vulnerable to nucleophilic attack; and an 'activating agent' is a substance that, in an aqueous mixture including a polyanionic polysaccharide, causes the polyanionic polysaccharide to become so activated;" and
- on page 6, lines 18-22, for the "second activated polyanionic polysaccharide" wherein it is described that "a modifying compound is defined as a reagent which, in the presence of an activated polyanionic polysaccharide, reacts with the activated carboxyl moiety of the polyanionic polysaccharide to form a new activated species capable of reacting with a nucleophile."

Applicants thus believe that this amendment does not introduce new matter.

Double Patenting Rejection

Claims 15-30 and 60-76 are newly rejected over claims 1-21 of US patent 5,760,200 or claims 1-4 of US patent 6,174,999 under the judicially created doctrine of obviousness-type double patenting both in view of US patent 4,713,448 to Balazs. Applicants have amended claim 15 to more clearly state that which they regard as their invention, as the Examiner has misapprehended the relevance of the teaching of Balazs to the scope of the instant claims with regard to the role of the modifying compound in the claimed process.

The presently claimed invention is directed to a process and articles made from this process, in which th process requires the modification of a polyanionic polysaccharide

(PAPS)(generally a water soluble starting material) with a combination of reagents into a water insoluble material. In the presently claimed combination of reagents that comprises an activating agent, a modifying agent and a nucleophile, the presence of the modifying agent permits the process to progress through an additional intermediate stage by allowing the formation of a second activated species (generally known in the art as an activated ester, thus the reference sometime to its carbonyl component) which is more reactive and selective toward the nucleophile than the first activated species formed between the polyanionic polysaccharide (or more precisely, its carboxylic moiety) and the activating agent (see specification at pages 13, line 26 to page 15, line 15.

The presence of the modifying agent as defined in this application permits the production of a second activated species more stable in acidic medium than the first species. This is particularly true when a carbodiimide is used as an activating agent, as the first activated species thus formed susceptible to nucleophilic attack, the *O*-acyl urea moiety, is also susceptible to rearrangement to an *N*-acyl urea moiety that is no longer susceptible to nucleophilic attack. Also applicants teach at pages 13 to 15, that the formation of this second activated species allows for a better control of the reactivity of the nucleophile, in particular if the nucleophile presents more than one nucleophilic group, by permitting the process to be conducted at lower pH values.

Balazs is applied in the instant rejection as supposedly teaching a process that involves treating hyaluronic acid with an aldehyde to produce a hyaluronic acid with an aldehyde covalently attached thereto. Reference is made to column 7 lines 39-42. Applicant traverses both the interpretation of the teaching of Balazs in the cited excerpt made by the examiner and its relevance in the claimed process involving the chemistry of carboxylic activation with activating agents.

Balazs teaches the formation of lightly cross-linked hyaluronan, which he refers as a hylan or HY, by reaction of formaldehyde during the extraction of hyaluronic acid from animal tissue. Although not specifically identified by Balazs, it would be evident to a person skilled in the art that once the carbonyl group of the formaldehyde has reacted with the hyaluronic acid to form the covalent cross-links, it most likely has been converted to a new structure, such as an acetal (see for example Shiurba et al., *Brain Research Protocols* 2 (1998) 109-119, copy attached

in appendix). On this point, Balazs hints that the formaldehyde is no longer present in its original form in the hylan product by always referring to it as "the combined formaldehyde." While Balazs refers to the incorporation of formaldehyde into the hylan product as a covalent attachment, Balazs does not state that such reaction leads to the formation of carbonyl group onto the hyaluronan. In particular, Balazs's description of the process for measuring the content of incorporation of formaldehyde (see col. 6, lines 47-54) involving boiling in sulfuric acid and distillation more closely agrees with the reverse hydrolysis of an acetal.

In any event, Balazs does not teach that the formaldehyde is reacted with an activated species of the starting hyaluronan, or that the hylan thus formed is rendered susceptible to nucleophilic attack, nor does it teach or suggest that such hylan may be reacted with a nucleophile to form a water-insoluble product. Thus applicants respectfully submit that the Balazs teaching are not properly combined with the teaching of the 5,760,200 and 6,174,999 patents, and that such combination does not teach the claimed invention as a whole.

Applicants respectfully submit that the present invention is patentably distinct and do not constitute double patenting over the5,760,200 and 6,174,999 patents and kindly request that these rejections be reconsidered and withdrawn.

JKF
CPG

THIS OPINION WAS NOT WRITTEN FOR PUBLICATION

The opinion in support of the decision being entered today (1) was not written for publication in a law journal and (2) is not binding precedent of the Board.

Paper No. 43

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

MAILED

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PAT. & T.M. OFFICE
BOARD OF PATENT APPEAL
AND INTERFERENCES

Ex parte ROBERT J. MILLER and XUEJIAN XU

Appeal No. 1995-2560
Application 07/833,973[1]

ON BRIEF

Before WINTERS, GRON, and ROBINSON, Administrative Patent Judges.

GRON, Administrative Patent Judge.

DECISION ON APPEAL UNDER 35 U.S.C. § 134

This is an appeal under 35 U.S.C. § 134 from an examiner's final rejection of Claims 15-17, 19, 22-30, 50-52, and 70-83. The examiner has indicated that Claims 8, 31-35, 37, 38, 44-49,

[1] Application for patent filed February 11, 1992. According to applicants, this application is a continuation-in-part of Application 07/703,254, filed May 20, 1991, now abandoned; which is a continuation-in-part of Application 07/543,163, filed June 25, 1990, now U.S. Patent 5,017,229; which is a continuation-in-part of Application 07/100,104, filed September 18, 1987, now U.S. Patent 4,937,270.

and 60-69 "are now allowable" (Examiner's Answer (Ans.), pages 1-2, bridging sentence). The Examiner's Answer does not mention the final rejection of Claims 18, 20 and 21, the only other claims still pending in the application. Therefore, we assume that the examiner did not intend to maintain the final rejection of Claims 18, 20 and 21.

Introduction

Claims 15-17, 19, 22-30, 50-52 and 70-83 stand finally rejected under 35 U.S.C. § 103 in view of the teaching of Zaffaroni, U.S. Patent 3,998,974, patented December 21, 1976. Contrary to the examiner's statement that "Appellant's brief includes a statement that claims 8, 15-35, 37, 38, 44-52 and 60-83 do not stand or fall together" (Ans., p. 3, first full para.), Appellants expressly stated, at least with respect to the examiner's rejection of Claims 15-30, 50-52 and 70-83, that "these claims stand or fall together" (Appellants' Brief (Br.), p. 8, first sentence).[2] Process Claim 15 and product-by-process Claim 70 are the broadest claims on appeal. They are reproduced below.

15. A method for making a water insoluble biocompatible composition, said method comprising

[2] It is not clear from the Examiner's Answer why the examiner did not maintain the rejection of Claims 18, 20 and 21 under 35 U.S.C. § 103 in view of Zaffaroni's teaching.

combining, in an aqueous mixture, one or more polyanionic polysaccharides, a modifying compound, a nucleophile, and an activating agent under conditions sufficient to form said composition wherein said modifying compound causes the formation of a new active carbonyl groups on said polyanionic polysaccharide.

70. A water insoluble composition prepared according to the method of claim 15 or 16.

Discussion

Claims 15-17, 19, 22-30, 50-52, and 70-83 stand rejected under 35 U.S.C. § 103 in view of Zaffaroni's teaching. The examiner explains (Ans., pp. 5-6, bridging para., through pp. 6-7, bridging para., repeated verbatim at pp. 7-8, bridging para., through p. 9, first full para.):

Zaffaroni discloses nonnutritive flavor imparting compounds of the general formula (F-Z)n-C wherein F is an active flavor imparting agent, C is a controlling agent for transporting and essentially restricting absorption of the compound (F-Z)n-C in a biological environment, Z is a covalent bond for bonding F to C and n is at least one. Zaffaroni discloses that the group C include polymer and polymeric like material of naturally occurring and synthetic origin which include commercially available celluloses such as sodium carboxymethylcellulose (see column 14, lines 14 and 15). Zaffaroni further discloses methods whereby the covalent attachment of the flavor imparting agent to the polymer can be carried out. One method involve[s (sic)] forming covalent bonds by reacting a pendant carboxyl group of a flavor imparting molecule with a hydroxyl, amine, mercaptan group or the like on the other reactant, wherein activation of a carboxyl group can be effected by the reaction of a carboxyl group with various carbodiimides, carbodiimidazoles, Woodward's reagent and the like to form highly active intermediates capable of reacting with other groups in the presence of a solvent and under mild reaction conditions to yield the desired compounds (see column 16, lines 30-60). This method

> disclosed by Zaffaroni appears to be within the scope of the method claimed by the Appellants when the instant claimed polyanionic polysaccharide is carboxymethylcellulose and the activating agent is a carbodiimide. . . .
>
> The Zaffaroni Patent further discloses other ingredients that can be added to the nonnutritive flavor imparting compound which include the yolk of eggs, milk products, glutamic acid, glycine and alanine which are within the scope of the instant claimed nucleophiles disclosed in Claims 30 and 82 of the instant application which sets forth the nucleophile being selected from a group consisting of an amino acid amide, a monofunctional amine, an amino acid ester, an amino alcohol, and amino thiol, and amino phenol, an amino catechol, an amino acid, a salt of an amino acid, a peptide, and a protein.
>
> Also see column 25, lines 55-60, which discloses the flavor imparting compounds being combine[d, sic] with medicinals and pharmaceutical formulations including tablets, capsules, powders, lozenges, drops, elixirs, syrups, suspensions, oils, emulsions, and the like . . . .

Zaffaroni refers to the F flavor imparting or enhancing agent utilized to form the F group of his (F-Z)n-C compound as a "'flavor imparting agent', 'enhancer' or '<u>modifier</u>'" (Zaffaroni, col. 4, l. 7-8; emphasis added). "These . . . include aliphatic aromatics, heterocyclics, and other compounds with different chemical structures such as alkaloids, terpene hydrocarbons, amides, oximes, benzenoids, fused rings, esters, ethers, acids . . . " (Zaffaroni, col. 4, l. 41-45). Zaffaroni also teaches at column 16, lines 7-29, that the polymer may be made to react "with a triazinyl substituted with both a halogen that reacts with the polymer and a nucleophilic substituent that reacts with

a reactive functionality of the flavor imparting groups" (Zaffaroni, col. 16, l. 7-12) or "the flavor imparting group can be bonded to the polymer by conventional processes such as diazotization, by reacting an acyl halide, a carboxyl or anhydride group of a polymer with an amino, hydroxyl or sulfhydryl group integral with or bonded to a flavor imparting group in aqueous buffer media, inert organic or mixed solvents . . ." (Zaffaroni at column 16, lines 13-19).

We see no error in the examiner's determination that Zaffaroni generically describes a process comprising combining, in an aqueous mixture, one or more polyanionic polysaccharides, a modifying compound, a nucleophile, and an activating agent under conditions sufficient to form a composition wherein said modifying compound causes the formation of a new active carbonyl group on said polyanionic polysaccharide. However, appellants argue that "[w]ater solubility is an essential characteristic of the Zaffaroni compounds since this characteristic permits their use as food additives" (Br., p. 18, first para.). As support for the argument, appellants cite Zaffaroni's disclosure at column 1, lines 59-64, and column 2, lines 19-24.

The examiner responds that, because Zaffaroni contemplates a method comprising combining, in an aqueous mixture, one of appellants' representative polyanionic polysaccharides, a

modifying compound, at least one of appellants' representative nucleophile components, and at least one of appellants' representative activating agents, under conditions sufficient to form a composition wherein said modifying compound causes the formation of a new active carbonyl group on said polyanionic polysaccharide, Zaffaroni would have suggested the processes and products by processes appellants claim to persons having ordinary skill in the art. To the contrary, appellants argue that Zaffaroni's teaching would have led persons having ordinary skill in the art to make and use water soluble compositions for flavoring foods, not water insoluble gels or films for use in surgical procedures (Br., p. 20, first full para.).

We fault both appellants and the examiner for their superficial reading of Zaffaroni. Moreover, appellants would have us consider the patentability of processes for making gels and films and products made by processes which are designed to make gels or films (Br., p. 20, first full para.), even though (1) Claims 15-30, 50-52 and 70-83 "stand or fall together" (Br., p. 8, first full sentence), and (2) appellants' broadest claims are not limited to processes for making gels and films and products made by processes which are designed to make gels or films.

Appellants have not shown that the examiner erred in finding that certain specific components Zaffaroni suggests for use in performing the processes he discloses for the utility he discloses and the products produced by those processes are within the scope of components appellants teach are suitable for use in performing the processes they claim to make products for the utility they indicate. Nevertheless appellants argue that, unlike the final products made by processes comprising the steps of their claimed processes, the final products made by the processes described by Zaffaroni by combining what appears to be the same or substantially the same components are water soluble. If appellants' arguments are correct, our findings are inconsistent. In fact, they are not.

While we agree with appellants' argument that Zaffaroni's final products are all water soluble, Zaffaroni teaches that substantially water insoluble intermediate products which also are made by the processes he discloses must be converted to their water soluble form for use as flavor imparting agents. Thus, we find that Zaffaroni describes not only direct processes for making water soluble nonnutritive flavor imparting compounds but also indirect processes for making water soluble nonnutritive flavor imparting compounds by producing substantially water insoluble precursor or intermediate compounds and thereafter

chemically improving their water solubility. For example, Zaffaroni preliminarily states, "The compounds of the invention preferably are water soluble and in use pass through the length of the gastrointestinal tract without degradation and without being absorbed from said gastrointestinal tract into the body of the host" (col. 1, l. 27-31; emphasis added). Later, Zaffaroni teaches:

> The compounds of the invention bearing a basic group, such as amino or the like, can be converted to non-toxic acid addition salts having improved aqueous solubility to enhance their use in foods, beverages and medicines.

(col. 18, l. 4-8);

> The nonnutritive flavor imparting compounds and intermediates used to prepare same when bearing at least one carboxyl functionality can also be used in the form of their base addition salts that have improved solubilities in aqueous media and other carrier systems.

(col. 18, l. 26-30);

> The solubilities of the nonnutritive flavor imparting compounds, or of intermediates leading thereto, also can be regulated by acylating the free hydroxyl group of the compound or the polymer or both.

(col. 18, l. 58-62); and

> The hydroxyl group attached to a nonnutritive flavor imparting compound, a polymer or an intermediate can optionally be etherified to form ether derivatives that have desirable solubilities in various media, carriers, foods, beverages and medicines.

(col. 19, l. 34-38).

Appellants emphasize the distinction between the water insoluble products produced by the processes they claim and the water soluble products Zaffaroni uses as nonnutritive flavor imparting agents (Br., pp. 18-20). Had appellants considered all the teaching of the reference, they would have learned, as persons having ordinary skill in the art have learned from reading the entire prior art disclosure, that Zaffaroni discloses (1) methods for making water soluble nonnutritive flavor imparting compounds, and (2) methods for making substantially water insoluble precursor or intermediate compounds whose aqueous solubilities can be chemically improved for use as nonnutritive flavor imparting compounds.

Prior art must be considered for everything it would have disclosed to persons having ordinary skill in the art, including nonpreferred embodiments. In re Burckel, 592 F.2d 1175, 1179, 201 USPQ 67, 70 (CCPA 1979); In re Lamberti, 545 F.2d 747, 750, 192 USPQ 278, 280 (CCPA 1976). Here, as in In re Plockinger, 481 F.2d 1327, 1332, 179 USPQ 103, 106 (CCPA 1973):

> . . . [A]*ppellants* introduced the issue of criticality in order to rebut any prima facie case of obviousness established . . . . In order to determine the propriety of the rejection, this [Board] . . . must be able to examine the evidence to determine whether, and to what degree, the criticality contended for by appellants exists.

That the teaching of the primary reference upon which the examiner relies and the reasons the examiner provided in the Answer for maintaining the final rejection differ from the teaching in the same reference which we highlight on consideration of the teaching of the reference as a whole, and our basis for holding the subject matter claimed in this case unpatentable, is insufficient to stay our review of the examiner's final decision on unpatentability over the evidence on appeal. To quote Judge Markey writing for the court in In re Grose, 592 F.2d 1161, 1165, 201 USPQ 57, 61 (CCPA 1979), "We review the decision, not the reasoning . . . ." Accordingly, we affirm the examiner's decision to finally reject Claims 15-17, 19, 22-30, 50-52 and 70-83 under 35 U.S.C. § 103 in view of the teaching of Zaffaroni.

Conclusion

The examiner's decision to finally reject Claims 15-17, 19, 22-30, 50-52 and 70-83 under 35 U.S.C. § 103 in view of the teaching of Zaffaroni is hereby affirmed.

Appeal 1995-2560
Application 07/833,973

No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136(a).

Affirmed

Sherman D. Winters
Administrative Patent Judge

Teddy S. Gron
Administrative Patent Judge

Douglas W. Robinson
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

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ELSEVIER

Brain Research Protocols 2 (1998) 109–119

BRAIN RESEARCH PROTOCOLS

Protocol

Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections

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Abstract

Formalin fixation, the chemical process in which formaldehyde binds to cells and tissues, is widely used to preserve human brain specimens from autolytic decomposition [10,16,25,41]. Ultrastructure of cellular and mitochondrial membranes is markedly altered by vesiculation [16], but this does not interfere with diagnostic evaluation of neurohistology by light microscopy. Serious difficulties are encountered, however, when immunocytochemical staining is attempted. Antigens that are immunoreactive in unfixed frozen sections and protein extracts appear to be concealed or destroyed in formalin-fixed tissues.

In dilute aqueous solution, formaldehyde is in equilibrium with methylene glycol and its polymeric hydrates, the balance by far in favor of methylene glycol [16,41]. Carbonylic formaldehyde is a reactive electrophilic species well known for crosslinking functional groups in tissue proteins, nucleic acids, and polysaccharides [16–20,22,27]. Some of its methylene crosslinks are readily hydrolyzed. Others are stable and irreversible. During immunostaining reactions, intra- and inter-molecular links between macromolecules limit antibody permeation of tissue sections [16], alter protein secondary structure [26,34], and reduce accessibility of antigenic determinants [10]. Accordingly, immunoreactivity is diminished for many antigens. Tissues are rapidly penetrated by methylene glycol, but formaldehyde binding to cellular constituents is relatively slow, increasing progressively until equilibrium is reached [16,24]. In addition, prolonged storage in formalin may result in acidification of human brain specimens [42]. Low pH favors dissociation of methylene glycol into formaldehyde [16], further reducing both classical staining and antigen detectability [14,42].

Various procedures have been devised to counter the antigen masking effects of formaldehyde. Examples include pretreatment of tissue sections with proteases [2,7,23], formic acid [3,28], or ultrasound [43]. Recently, heating of mounted sections in ionic salt solution by microwave energy was found to restore many antigens [6,37,44–48,54–56]. Theory and practice of microwave antigen retrieval are covered extensively in the handbook *Microwave Cookbook for Microscopists* [29]. A concise overview of microwave methods in the neurosciences has been published [33], and clinical applications have been reviewed [30]. In this context, it should be noted that fresh tissues may be stabilized for immunocytochemistry by reversible, non-chemical binding processes such as cryosectioning after microwave treatment [12] and freeze-drying [52]. Thus, it may be possible to enhance immunostaining for some antigens by microwave irradiation of unfixed as well as fixed specimens.

Parameters to be optimized for microwave retrieval of specific antigens include temperature, irradiation time, tissue buffer composition, salt concentration, and pH [12,47,53]. Temperature, irradiation time, and pH are key variables [12,13]. With this in mind, an optimal method was developed for retrieval of a wide variety of antigens in human brain tissues [14]. Typical microwave protocols employ elevated temperatures that may reach 100°C, where denaturation causes irreversible uncoiling and disruption of protein secondary and tertiary structures [11]. Under these conditions, stable covalent bonds securing methylene crosslinks between polypeptides remain intact [20], but more reactive links formed by Schiff bases may be hydrolyzed [45]. Resultant conformational changes presumably expose buried loops of continuous amino acids and protruding regions [1], increasing accessibility of their epitopes [51].

Protein denaturation seems to be a reasonable explanation for the effects of microwaves on antigen retrieval. This idea is supported by the observation that denaturing solutions such as 6 M urea increase immunoreactivity of some antigens [8]. Still, the molecular basis of these effects remains unresolved, in part due to the complex chemistry of formaldehyde reactions with tissue constituents [41]. Indeed,

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some methylene bridges between similar groups such as NH_2 and NH may be hydrolyzed by washing fixed tissues in distilled water at ambient temperature for several weeks [24,41]. Moreover, denaturation by conventional heating enhances antigenicity as well as classical neuromatomical staining of formalin-fixed tissue [15,39,40,49]. When such externally heated specimens are immunostained and viewed by light microscopy, the results are almost indistinguishable from those obtained by microwave irradiation [58]. Nevertheless, the current widespread use of microwave methods in clinical and basic science laboratories likely results from the speed, convenience, and reproducibility of the results.

Loss of immunoreactivity for many antigens likewise may occur when tissues are dehydrated in alcohol before they are embedded in paraffin. Exposure to alcohol causes antigenic denaturation, but clearing in xylene and heating in liquid paraffin do not [52]. While there are numerous reports of microwave procedures for formalin-fixed, paraffin-embedded tissues [6,8,9,35,36,38,44,45,48,57,60], alternative methods that would further extend the range of retrievable epitopes have received less attention. One simple approach may be to irradiate free-floating sections with microwaves. A previous study found that heating vibratome sections in solution with microwaves resulted in severe wrinkling of the tissues. This problem was avoided by irradiation of tissue slices in buffer prior to sectioning [13]. Another strategy is presented here for neuropathological studies of human brain [50]. Vibratome sections in isotonic, mildly acidic citrate buffer [6,55] are heated to the boiling point with microwaves. After brief denaturation at 100°C, the sections are simmered for 5 min. To remove wrinkles, sections are incubated subsequently in Tris-buffered saline containing serum proteins and non-ionic detergent. The method also is suitable for single- and double-labeling studies of neural antigens in formalin-fixed tissues from experimental animals. © 1998 Elsevier Science B.V.

Themes: Cell biology

Topics: Staining, tracing, and imaging techniques

Keywords: Immunocytochemistry; Microwave antigen retrieval; Neuropathology; Tau protein kinase I/glycogen synthase kinase-3β

1. Type of research

Neurobiology and neuropathology.

2. Time required

- Microwave procedure: 1 h.
- Single-label immunostaining: 24 h.
- Double-label immunostaining: 48 h.
- Nissl counterstaining: 45 min.
- Thioflavin-S counterstaining: 45 min.
- Subbing of glass microslides: 24 h.

3. Materials

Unless otherwise specified, all chemical reagents are available from Sigma Chemical Co. (St. Louis, MO) and all antibody reagents are from Vector Laboratories (Burlingame, CA). Ethanol, methanol, and xylene are ACS reagent grade. Aqueous solutions are prepared with deionized water. Citrate buffer is 50 mM trisodium citrate dihydrate, pH 6.0. Antibody diluting buffer (DB) is 20 mM Tris-HCl, pH 7.4, 1% normal goat serum, 2% bovine serum albumin, 0.4% Triton X-100, 150 mM sodium chloride. Tris-buffered saline (TBS) is 20 mM Tris-HCl, pH 7.6, 150 mM sodium chloride.

- Special equipment:
  - Beakers (Pyrex® glass, 100 ml).
  - Coverslips (24 × 50 mm, Corning).
  - Lids from high density polyethylene Coplin jars (8541, Thomas Scientific).
  - Micropipettes and disposable tips for 1 ml, 200 μl, and 20 μl volumes.
  - Microwave oven (e.g., 500–1000 W) with an autodefrost mode.
  - Millex-GV filters (0.22 μm, SLGV025LS, Millipore Corp.).
  - Paint brushes (Sable brushes, 11812, Ted Pella Inc.).
  - Pens (Marker II/Superfrost black ink, Securline, Precision Dynamics Corp.).
  - Rocking platform (e.g., Reliable Scientific).
  - Slides (FISHER*finest* Premium glass, 12-544-2, Fisher Scientific).
  - Staining dishes (Wheaton 180 glass, 20 slide, S 6141, Sigma Chemical Co.).
  - Syringes (disposable plastic, 5 ml).
  - Tissue culture dishes (35 × 10 mm, Falcon 3001, Becton Dickinson).
  - Tissue culture plates (six-well, Falcon or Becton Dickinson).
  - Vibratome (Series 1000, Pelco 101, Ted Pella Inc.).
- Chemicals and reagents:
  - Acetic acid.
  - Bovine serum albumin (fraction V powder, A 2153).
  - Cresyl violet acetate (C 1791).
  - Chromium potassium sulfate (C 5926).
  - Ethanol.
  - Formic acid (F 0507).
  - Gelatin (Type B from bovine skin, approx. 75 Bloom, G 6650).
  - Hydrogen peroxide (30% stock solution, H 1009).
  - Methanol.
  - *N,N*-Dimethylformamide (D 8654).

- Permount coverslip mounting medium (Fisher Scientific).
- Sodium acetate (S 8625).
- Sodium chloride (S 9888).
- Sodium citrate (S 4641).
- Thioflavin-S (C.I. 49010, Direct Yellow 7, T 1892).
- Triton X-100 (X-100).
- Trizma base (T 1503).
- Immunochemicals:
  - Biotinylated anti-mouse IgG (H + L) (BA-2000).
  - Biotinylated anti-mouse IgM (μ chain specific) (BA 2020).
  - Biotinylated anti-rabbit IgG (H + L) (BA-1000).
  - Biotinylated anti-sheep IgG (H + L) (BA-6000).
  - DAB Substrate Kit for Peroxidase (SK-4100).
  - SG Substrate Kit for Peroxidase (SK-4700).
  - Vectastain ABC Kit (Standard) (PK-4000).
  - VIP Substrate Kit for Peroxidase (SK-4600).

4. Detailed procedure

4.1. Microwave irradiation of free-floating brain sections

A. Cut 40 μm vibratome sections of formalin-fixed brains, and float them in 2 ml of TBS (20 mM Tris-HCl, pH 7.6, 150 mM sodium chloride) in a multiwell tissue culture plate. A six-well plate (well diameter approx. 3.5 cm) works best, because each well can easily hold six or more tissue sections (1 × 2 cm) in a volume of 1–2 ml. The identity of each section can be determined by matching its shape to an extra section cut and floated in TBS in a correspondingly labeled Petri dish or multiwell plate. Alternatively the shape of each section can be drawn in a notebook for later identification with its corresponding case number. Label the lid of the plate above each well to indicate the antibody, treatment conditions, or other information. In order to confirm that antigen retrieval occurs, immunocytochemical staining of microwave-irradiated sections should be compared to that of adjacent sections that are not subjected to microwave heating; immunoreactivity should be absent in these negative controls. Extra sections should be cut for this purpose. If fresh tissue is available, conventional frozen sections may also be prepared. Store vibratome sections in TBS overnight at 4°C. If serial sections are needed, number the wells on the plate lid accordingly. Set up an additional plate with sections from each case for classical histological stains such as hematoxylin and eosin, Nissl, thioflavin-S, and Bielschowsky silver.

B. Just before use, prepare a 1:100 dilution of a 30% hydrogen peroxide stock solution in methanol. To inactivate endogenous peroxidase activity in the tissue sections, remove the TBS from each well with a micropipette and add 2 ml of methanol/0.3% hydrogen peroxide for 30 min. Set the plate on a rocking platform during this time. Slow, gentle rocking is sufficient for all subsequent incubation and washing steps. Rapid rocking may result in sections sticking to the sides of the wells or solutions spilling from one well into another. Sections will shrink and wrinkle in methanol due to dehydration. These distortions are only temporary, however.

C. Discard the methanol/hydrogen peroxide. Rinse briefly in TBS, and wash the sections in 2 ml of TBS by rocking the plate for 10 min three times to remove all traces of methanol. This will also allow time for rehydration and expansion of the sections. Wrinkles will gradually disappear.

D. Transfer the vibratome sections to 100 ml Pyrex® glass beakers, each containing 80 ml of citrate buffer (50 mM trisodium citrate dihydrate, pH 6.0) [6.55]. The isotonic 150 mM sodium ion concentration of this buffer will prevent shrinkage or expansion of the sections during heating. It is convenient to mark each beaker with an alcohol soluble ink pen to match its corresponding well in the tissue culture plate. Those beakers that do not contain tissue sections can be filled with 80 ml loads of water. To distribute the heat evenly and prevent overheating, six beakers should be irradiated at the same time. Cover each beaker with the inverted lid from a high density polyethylene Coplin jar (8541, Thomas Scientific), concave side down. Since the lid is not attached to the beaker, some steam can escape but most will condense and drip back into the beaker. Never screw lids, even loosely, on containers that are microwaved [33].

E. Members of the European Microwave Organization have established guidelines for reporting methods of microwave irradiation [31]. Accordingly, in this work [50] we used a Tappan Speed Wave 1000 microwave oven (USA Model No. 56-5472-10/03, serial number HG 30804110, product No. 941354362, Manufacture date February 1993, 120 V, 60 Hz, output 1000 W, frequency 2450 MHz). The total irradiation time was 7 min. Beakers were positioned symmetrically around the center of the rotating platform of the microwave oven. They were set on paper towels in a microwave-safe plastic dish to catch any liquid that escaped. Irradiation was performed at full power until the buffer just began to boil. This took about 2 min. Then the power was turned off, and the setting was changed to autodefrost mode (i.e., intermittent microwaves at 200–300 W, cycling on for 5–10 s every 30 s). The timer was set for 5 min, and the beakers were irradiated again. During this period, the temperature of the simmering liquid remained between 95 and 100°C, and the liquid did not boil over. To confirm the temperature, the liquid in each beaker was measured with a thermometer just after the microwave irradiation stopped.

F. Safety precautions should be strictly observed as described [33]. To avoid thermal injury, wear insulated gloves and safety glasses when handling hot liquid containers. Remove the beakers from the microwave, take off the lids, and allow the solution to cool on the lab bench for

approx. 30 min until the temperature falls below 40°C as measured by a thermometer.

G. Using a small, soft bristle paint brush (11812, Ted Pella Inc.), transfer the sections to a multiwell plate containing TBS, and carefully unfold them to remove creases and wrinkles. Wash the sections for 5 min two times in 2 ml of TBS on a rocking platform (e.g., Reliable Scientific).

4.2. Immunocytochemistry of vibratome sections

H. This part of the protocol is a modification of the method of Cataldo et al. [4,5]. Remove the TBS, and wash the sections in 2 ml of diluting buffer (DB; 20 mM Tris, pH 7.4, 1% normal goat serum, 2% bovine serum albumin, 0.4% Triton X-100, 150 mM sodium chloride) by gently rocking for 10 min three times. As the sections absorb protein in the presence of non-ionic detergent, wrinkles will gradually disappear. Three hundred ml of DB is generally enough for an experiment involving two six-well plastic tissue culture plates. Prepare DB as follows. (1) Dissolve 6 g of bovine serum albumin in 150 ml of TBS by magnetic stirring for approx. 20 min. (2) To prevent micelle formation, dissolve 1.2 ml of Triton X-100 into a separate 150 ml volume of TBS for approx. 20 min. (3) When solutions are completely dissolved, combine and mix them by magnetic stirring for 5 min. (4) Using a 5 ml syringe, sterile filter 3 ml of normal goat serum into the combined solution. Use a Millex-GV 0.22 μm filter (SLGV025LS, Millipore Corp.) or equivalent filter. (5) Filter the solution through Whatman No. 1 paper in a large funnel, and store it at 4°C to inhibit microbial growth.

I. Remove the DB, and add 2 ml of sterile-filtered 20% serum (e.g., normal goat serum in TBS) for 1 h to block non-specific protein binding sites in the tissue sections. Then, continue the single-label or double-label immunostaining procedures as described below.

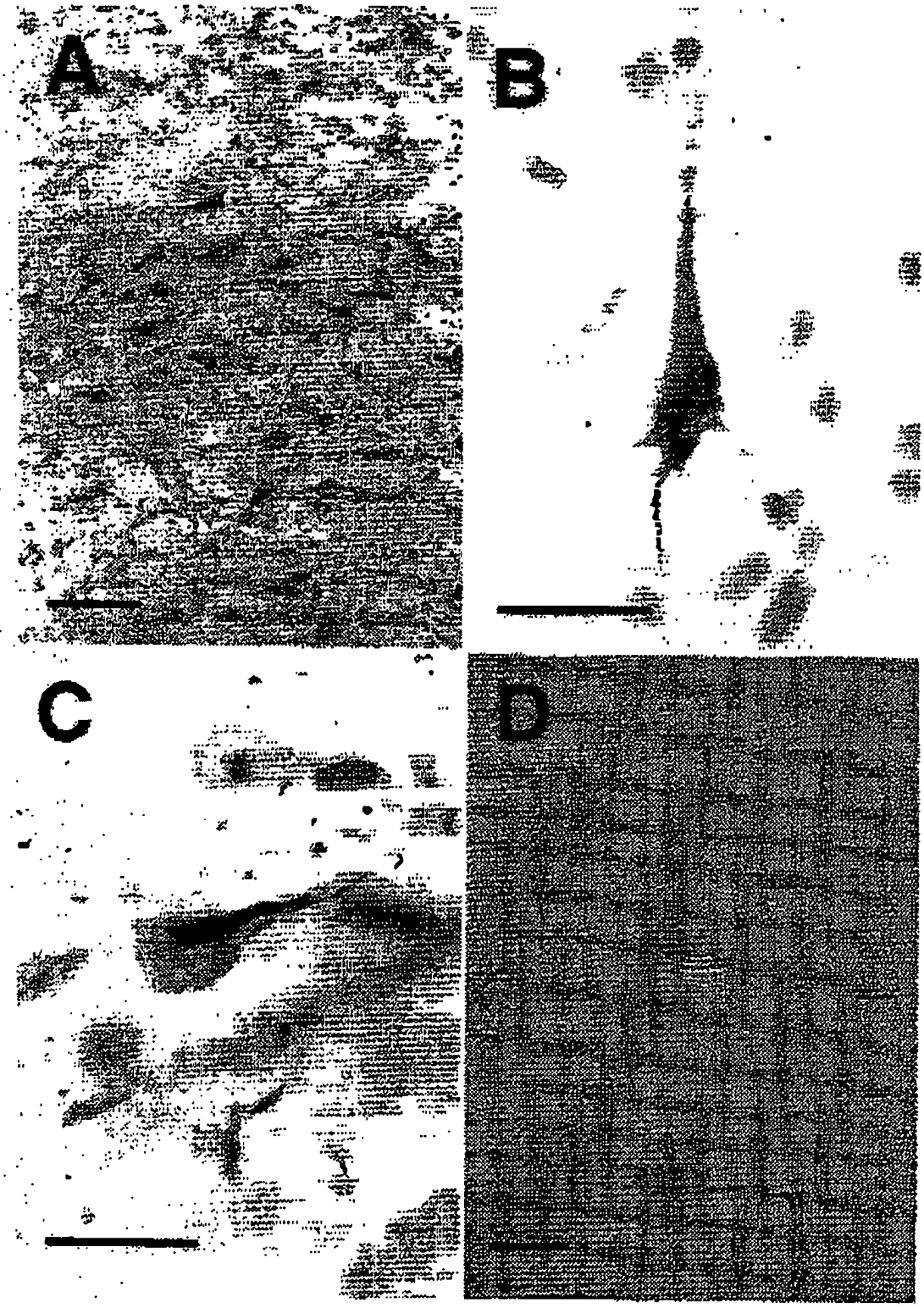
J. For best results, centrifuge all antibody stock solutions (primary and secondary) at 15000 rpm (e.g., TOMY MTX-150 centrifuge) in microfuge tubes for 5 min just before preparing working dilutions. This will remove partially denatured antibody aggregates and precipitates that non-specifically bind to the tissue sections, increasing the background staining. Incubate sections in 1 ml of an appropriately diluted primary antibody in DB. Serial dilutions should be tested and the immunoreactive endpoint determined. Incubation time (several hours versus overnight) and temperature (room versus 4°C) may vary according to experimental conditions. High dilutions of antibody and long incubation times at 4°C are preferred to attain a low non-specific background stain. For example, rabbit antisera at high dilution (e.g., 1:2500) usually require an incubation time of 18–20 h and temperature of 4°C to prevent microbial growth. When rabbit or mouse antibodies are in the form of purified IgG, the dilution is in the range 1–10 μg/ml. Always dilute antibodies in DB or TBS, never water. Negative controls consist of sections incubated with pre-immune serum, normal IgG, or TBS in place of primary antibody. In cases where anti-peptide antibodies are used, controls for specificity should include antibodies preabsorbed overnight at 4°C with 10–100 μg/ml of their peptide immunogen or with the same amount of a heterologous peptide.

K. Wash sections for 10 min three times in 1 ml of DB as before. Incubate the sections in 1 ml of biotinylated secondary antibodies in DB for 1 h. This is affinity-purified biotinylated anti-rabbit IgG (H + L) produced in goat if the primary antibody is polyclonal rabbit antiserum or IgG. It is affinity-purified biotinylated anti-mouse IgG (H + L) produced in horse if the primary antibody is a mouse monoclonal IgG. Centrifuge the solution as described in step J, and add 50 μl to each 10 ml of DB. In some cases (e.g., when mouse monoclonal IgM is used as primary antibody), a biotinylated anti-IgM secondary antibody will be needed.

L. Wash for 10 min three times in 1 ml of DB. Incubate the sections in 1 ml of avidin-biotin complex (ABC) for 1 h. Use a standard Vectastain ABC Kit. The ABC reagent is made by adding two drops of solution A and two drops of solution B to 10 ml of TBS at least 30 min before use. It is usually convenient to mix the ABC complex 20 min into the secondary antibody incubation step.

M. Wash for 10 min three times in 1 ml of TBS. DAB is a potential carcinogen, so wear disposable gloves during this part of the procedure. Use the DAB Substrate Kit for

Fig. 1. Immunocytochemistry of anti-tau protein kinase I/glycogen synthase kinase-3β in the human hippocampal CA1 subfield [50]. Forty μm thick vibratome sections of brain tissue were pretreated with microwaves and processed for immunocytochemistry as described in Section 4. A, C, and D are Alzheimer's disease brains. B is a control brain with Alzheimer-like changes. A, B, and D are single-label DAB (brown) immunostains. A and B are counterstained light blue with cresyl violet to visualize nuclei and Nissl substance in cell bodies. C is a double-label DAB (brown)/VIP (purple) immunostain without a counterstain. D is counterstained with thioflavin-S (yellow) and viewed simultaneously in tungsten and ultraviolet light. Scale bars = 80 μm (in A), = 40 μm (in D), and = 30 μm (in B,C). These digital images were scanned from 35 mm color slides and processed at a Macintosh workstation as described [50]. A: anti-tau protein kinase I/glycogen synthase kinase-3β reacts with a focal group of neurofibrillary tangle bearing pyramidal neurons. Note that the strong staining of these cells and their surrounding neuropil stands out against the weaker, uniform reaction of the neighboring neuropil and cell bodies. B: anti-tau protein kinase I/glycogen synthase kinase-3β localizes in a pyramidal cell body, apical dendrite, and basal neurites including the proximal axon in a control brain with Alzheimer-like changes. C: anti-tau protein kinase I/glycogen synthase kinase-3β immunoreactivity (brown) is localized adjacent to an early neurofibrillary tangle stained by anti-tau phosphoserine 413 (purple) in a pyramidal neuron cell body. D: anti-tau protein kinase I/glycogen synthase kinase-3β reacts with a globular cluster of distended neurites (brown) at the periphery of a neuritic plaque with a thioflavin-S histofluorescent β-amyloid protein core (yellow).



Peroxidase (SK-4100, Vector Laboratories) to stain the sections. Prepare the chromogen solution just before use. It is important that the squeeze bottles in this kit be held at a 45° angle while dispensing the solutions to insure that the droplet size is correct. The final solution should have a purple color. A yellow color indicates that the solution is weak, and it should be discarded. Sections should be separated from each other and unfolded with a small paint brush just prior to staining to permit even penetration of the chromogen. Use a light background (e.g., white paper) beneath the dish to better visualize the staining process. Be sure that the sections are completely immersed. Sections will turn light to dark brown depending on the amount of non-specific background immunoreactivity. Do not allow sections to get too dark. Two to 3 min of staining is usually optimal. To terminate the reaction, transfer each section with a small paint brush to a matching well in a second culture plate containing 2 ml of deionized water per well. Decontaminate the remaining DAB solution with household laundry bleach and discard it as toxic liquid waste. Keep separate paint brushes for use with DAB solutions. Wash them in water, but do not treat them with bleach.

N. Wash sections for 5 min by transferring them to a new six-well plate with wells containing 2 ml of deionized water. Remove the water with a micropipette, discard it in the toxic waste, and wash the sections two more times for 10 min in 2 ml TBS. For double-labeling immunocytochemistry, elution of antibodies and avidin-biotin-peroxidase complexes between the first and second labeling reactions is accomplished by washing the sections for 10 min three times in 50 mM Tris-HCl, pH 7.6, containing 10% dimethylformamide [59]. Remove the dimethylformamide solution, and wash the sections for 10 min in 2 ml of TBS three times. Then, re-block with 20% serum (step I), and repeat steps J–M with the second primary antibody. Use a VIP (SK-4600, Vector Laboratories) or a SG (SK-4700, Vector Laboratories) Substrate Kit for Peroxidase as the chromogen for the second primary antibody. DAB polymers are insoluble in dimethylformamide, making them ideal as the first chromogen. Purple VIP or blue-grey SG reaction products provide excellent contrast with brown DAB precipitates. These reaction products are also resistant to the ethanol and xylene used to dehydrate the sections prior to mounting permanent coverslips. Double-label controls may include reversing the order of antibody staining as well as substituting rabbit anti-GFAP IgG (Dako, USA), recognizing an astrocyte-specific intermediate filament protein, for the secondary antibody.

O. Some practice is required for this step, especially if one intends to mount several sections per slide. Using a small soft bristle paint brush and a Petri dish filled with TBS, pick up the sections on gelatin-subbed glass microslides. See Section 6.2.4 for preparation and storage of these slides. Drain the excess liquid from the slide by holding it vertically on Kimwipes®, and place it face up on a flat surface. Small wrinkles will smooth out as the section slowly shrinks and dries. For best results, air-dry the sections for at least 1 h, usually overnight, at room temperature to firmly affix them to the slides.

P. Based on the shape of each section, identify the case number, and label the frosted end of each slide with a Marker II/SuperFrost black ink marking pen (Securline, Precision Dynamics Corp.). Single-labeled sections may be counterstained with cresyl violet to visualize nuclei and Nissl substance. To avoid color mixing, double-labeled sections should not be counterstained. Optional protocols for demonstrating nuclei and Nissl substance or amyloid to compare with the tissue distribution of single-label immunostains are detailed in Sections 6.2.2 and 6.2.3. Dehydrate the sections and mount coverslips as described in Section 6.2.2, part C.

5. Results

5.1. Immunohistology

It should be noted that the immunostaining protocol presented here is not universally applicable to all tissue antigens masked by formalin fixation. Results may vary according to the specificity and avidity of the primary and secondary antibodies that are employed. In addition, a certain amount of trial and error is required to optimize primary antibody dilutions, temperature, and microwave irradiation times as well as other variables such as pH and ionic salt concentration [14,47,53]. In our experience (Fig. 1A–D), however, anti-peptide antibodies to developmentally regulated protein kinases gave excellent results that were specific when compared to antibody preabsorption controls [50]. Though the immunoreactivities of masked epitopes are often restored and morphology is generally well preserved, Nissl staining is reduced, and membranes may appear less distinct. In addition, it is well known that there is considerable individual variation in the immunoreactivity of human autopsy brain tissues, resulting in part from autolysis that takes place during the post-mortem interval between clinical death and removal of the brain. During this interval, proteases and nucleases take their toll, and some antigens are completely degraded. In addition, tissues may become acidified by prolonged storage in formaldehyde, leading to decreased immunoreactivity as discussed below. Nevertheless, the enigmatic nature of chronic neurodegenerative diseases such as Alzheimer's disease, for which there are currently no good animal models of pathogenesis, leaves little alternative than to make the best use of available autopsy material for research. These methods may prove useful to those who undertake such investigations.

6. Discussion

6.1. Troubleshooting

Problems are rarely encountered when these procedures are followed to the letter. Of course, deviation from the script, no matter how trivial or well intentioned the change may seem, will create frustrations in pinpointing the source of any trouble. In our work [50], we used human brain specimens that were fixed in neutral buffered formalin for less than 1 year. Specific antigen immunoreactivity was demonstrated after vibratome sections were heated with microwaves in isotonic citrate buffer at pH 6.0 [6,55]. These conditions were only mildly acidic and may not be suitable for all antigens. Long-term exposure to formaldehyde fixative results in progressive tissue acidification that may decrease both classical and immunocytochemical staining properties [42]. Such tissues require special treatments for antigen retrieval. Microwave heating or boiling the tissues in basic buffer is one possible solution to the problem [42], and this approach has been optimized recently [14].

Microbial growth in the antibody diluting buffer may occur occasionally, but this is quickly recognized when sections are stained with cresyl violet for Nissl substance. Bacterial clusters often form blue blotches on the sections, and individual bacteria are clearly visible in the light microscope. Contamination may be averted by preparing proteinaceous solutions fresh for each staining experiment, if possible just before use, then filtering and storing them at 4°C at all times. Questions regarding the immunodetection and substrate kits should be referred to the manufacturer's technical support service and troubleshooting guide, both of which are excellent. Serial dilutions of all primary antibodies should be tested and their immunoreactive endpoints determined. Likewise, in order to avoid overstaining that may obscure antibody specificity, minimum development times in chromogen solutions are preferable. Antibody stock solutions should be centrifuged before working dilutions are aliquotted. This will remove aggregates and precipitates of partially denatured antibodies that may increase non-specific background staining, which is most conspicuous over white matter tracts but not confined to them. Once air-dried on gelatin-subbed slides, sections should never detach during counterstaining and mounting procedures. If they do, check the source and concentration of gelatin in the subbing solution as well as the method used to coat the slides. Finally, patient consistency in performance is the best insurance against a major mishap.

6.2. Alternative and support protocols

6.2.1. Internet resources

The following Internet websites contain a wealth of information that may be useful for troubleshooting as well as designing new applications for microwave histotechnology:

- Advances in Temperature Control of Microwave Immunohistochemistry (http://www.ebsciences.com/papers/mw_temp.htm)
- Development of Microwave Immunohistochemistry (http://www.ebsciences.com/papers/mw_immun.htm)
- Microwave Antigen Retrieval Technique (http://www.ebsciences.com/papers/antigen.htm)
- Microwave Irradiation of Cryostat Sections Accelerates and Improves Nitric Oxide Synthase Staining (http://www.ebsciences.com/papers/Microlabs.htm)
- Microwave Processing Techniques for Microscopy (http://www.ebsciences.com/papers/mw_tech.htm)
- Special Stains Using the Microwave (http://www.ebsciences.com/papers/moore.htm)
- Tips and Tricks of Microscopy (http://www.biotech.ufl.edu/~emcl/tips.html)

6.2.2. Nissl counterstain for single-label immunocytochemistry

Identification of various types of neurons and glia in human brain tissues is greatly assisted by assessment of nuclear morphology and location within specific laminae. In general, neurons have a relatively large nucleus and cytoplasm as well as abundant ribosomes compared to glia. Counterstaining for Nissl substance with cresyl violet, which stains both neuronal and glial nuclei, offers the advantage of being able to correlate single-label immunostaining with neural cell type and tissue architecture. This is especially useful when DAB, which does not non-specifically stain nuclei, is the immunoperoxidase chromogen substrate (Fig. 1A and B). The brown color of its reaction product is unchanged by the Nissl stain described here. In addition, the counterstain can be completely removed by immersing the exposed section in acid-alcohol for 10 min, permitting further studies after photomicroscopy. As Nissl substance is very sensitive to nucleases, its presence or absence also reflects the extent of tissue autolysis at the time of formalin fixation. However, in the case of double-label immunostaining (Fig. 1C), especially when the chromogen may tinge nuclei, or when chromogen colors may be altered by subsequent Nissl staining, it is advisable to omit such counterstaining.

A. Wheaton 180 glass horizontal style slide staining dishes (S 6141, Sigma Chemical Co.) can be used to set up all of the various alcohol and xylene solutions used in this procedure. Load immunostained, air-dried sections on gelatin-subbed microslides (25 × 75 × 1 mm) into bottomless glass racks with wire handles. Be sure that all sections are attached to the slide surface nearest you and that the frosted end of each slide is on the left. This will prevent you from accidentally wiping off one of the sections when you pick up a slide from xylene to mount the coverslips. Insert one slide per slot (i.e., ten slides per rack) to allow

enough room for free flow of liquid across the surface of the sections during the dipping procedure described below. Rehydrate the sections in water for 2 min. Counterstain sections with freshly prepared cresyl violet staining solution for 20 min at room temperature. Stock solutions are prepared as follows.

- *Cresyl violet acetate.* Dissolve 0.2 g of cresyl violet acetate (C-1791, Sigma Chemical Co.) in 150 ml of deionized water. Filter (0.22 μm) and store at 4°C.
- *CV buffer, pH 3.5.* Mix 94 ml of 0.1 M acetic acid (0.6 ml of concentrated acetic acid per 100 ml of water) with 6 ml of 0.1 M sodium acetate (1.36 g per 100 ml of water).
- *Cresyl violet staining solution.* Just prior to staining, mix 12 ml of cresyl violet stock solution with 200 ml of CV buffer, pH 3.5.

B. Rinse in two changes of water by dipping the slide rack up and down ten times to remove excess stain. If the sections have not been treated with microwave irradiation, destain them briefly in acid-alcohol (200 ml of 70% ethanol in water plus five drops of concentrated acetic acid) by slow magnetic stirring and dipping ten times. Please note that microwave treatment reduces the affinity of the tissue for the Nissl stain. In order to compensate for this effect, the usual destaining step in acid-alcohol should be omitted.

C. Rinse in two changes of water by similar dipping of the rack ten times to remove the acid-alcohol. Then, dehydrate the sections by dipping ten times in each of the following alcohol solutions: 50%, 70%, 95%, and 100% ethanol in water. Finally, dip the slides in two changes of xylene, and leave the rack in xylene. Remove each slide with a forceps, wipe off the xylene from the back, and mount a coverslip (24 × 50 mm) over the section, being careful to gently press out all of the excess mounting medium (e.g., Permount, Fisher Scientific) with the forceps. Dip the slide by hand ten times in xylene to remove excess mounting medium. Wipe the back and edges of the slide while holding the coverslip in place with thumb and forefinger. Place the slides face up on paper towels to dry overnight. Do not load them vertically into slide boxes while wet.

D. Grossly, the sections should be almost transparent except for the areas of specific immunostaining. Immediately after mounting the coverslips, examine the slides with a microscope. The nucleoplasm should be clear except for a densely stained nucleolus and scattered chromatin. The nuclear membrane should be sharply delineated. The cytoplasm should also be clear except for ribosomes whose staining should be punctate blue or violet. There should be no additional blue background staining (e.g., white matter tracts which contain no ribosomes). If the counterstaining is too light or too dark, reload the slide rack and immerse it in xylene until the coverslips fall off. This usually takes 20–30 min. Then dip the rack in xylene and transfer the rack back through the ethanol series (100–50%), again dipping ten times in each solution. Finally, dip in two changes of deionized water to remove alcohol. Repeat the cresyl violet counterstain for 20 min, and destain for a shorter or longer time in acid-alcohol as needed to adjust the stain intensity.

6.2.3. Thioflavin-S amyloid counterstain for single-label immunocytochemistry

This protocol is a modification of those previously described [5,21]. Along with neurofibrillary tangles and neuropil threads, amyloid plaques are histopathological hallmarks of Alzheimer's disease. Plaques are composed of proteinaceous extracellular amyloid deposits in the β-pleated sheet conformation. Thioflavin-S is an intercalative fluorescent dye that has proved useful for identifying such conformations in formalin-fixed tissue proteins. Though it does not identify all forms of amyloid deposits, it remains a quick and reliable estimate of the amyloid burden in classical neuritic plaques, and its yellow-green color contrasts well with the brown color of DAB reaction products. Thus, DAB works best as the chromogen for the single-label immunostain (Fig. 1D). Some intracellular neurofibrillary tangles, especially those in later stages of development, are also stained by thioflavin-S, but their size and shape make them easy to distinguish from amyloid plaques. However, thioflavin-S permanently tinges the tissue yellow, making it difficult to use for subsequent histochemical procedures.

A. Air-dry immunostained vibratome sections on gelatin subbed slides as usual. Do not counterstain sections with cresyl violet (Nissl) or other histological stains. Prepare 1% thioflavin-S (C.I. 49010, Direct Yellow 7, T1892, Sigma Chemical Co.) by dissolving in 70% ethanol for 1–2 h while stirring. Using a large Büchner funnel and vacuum, filter the solution through Whatman No. 6 paper or equivalent. Practical grade reagent dissolves slowly and incompletely. Consequently, it also filters slowly, and some particulate matter may remain suspended in solution. Store the stock solution in a brown glass bottle in the dark at 4°C.

B. Rehydrate sections in a graded ethanol series (100%, 95%, 70%, and 50% ethanol in water) for 3 min in each solution. Immerse sections in 1% thioflavin-S in 70% ethanol for 20–30 min in the dark. Return the solution to the stock bottle, and save it for future use.

C. Wash sections for 3 min three times in 70% ethanol to remove excess dye. Dehydrate sections by dipping ten times in each solution of a graded ethanol series (50%, 70%, 95%, and 100% ethanol in water).

D. Dip sections ten times in each of two changes of xylene. Mount coverslips (Permount). Store slides in the dark until use.

E. Examine sections in a fluorescence microscope (e.g., Zeiss Axioskop) under ultraviolet light using a thioflavin-

specific filter. Amyloid fluoresces yellow-green, DAB immunoperoxidase reaction products appear brown, and other tissue components are reddish when tungsten light is added simultaneously.

6.2.4. Subbing glass microslides with gelatin

This protocol is for standard use with vibratome sections. A special protocol must be used for autoradiographic preparations. Use 25 × 75 × 1 mm FISHER*finest* Premium microscope slides (12-544-2, Fisher Scientific) or equivalent. Powdered gelatin may be obtained from Sigma Chemical Co.

A. Load glass slides into bottomless stainless steel racks, each holding about 30 slides. Be careful to touch only the edges of the slides while handling them. Using a wire handle, immerse each rack in 95% ethanol for 5 min. Then transfer the slides to a basin of running tapwater for 5 min, and immerse them again in distilled water for 5 min two times. Dip the racks up and down ten times between each change. Cover the shelves of a large 37°C oven with aluminum foil to catch drippings from the subbing solution. Place the racks in the oven to dry them completely overnight. Also, prewarm a large glass staining dish that will be used to contain 500 ml of subbing solution.

B. The next morning, prepare the subbing solution just prior to use as follows. Add 3 g of gelatin to 500 ml of deionized water prewarmed to 50–60°C while magnetically stirring on a hot plate. Do not overheat the solution, as this will denature the protein. Cool the straw colored solution to 45°C, and add 0.3 g of chromium potassium sulfate. The solution should then acquire a greenish tinge. To remove bubbles and particulate matter, filter the solution into the prewarmed staining dish inside the oven. Use a 27 cm diameter Whatman No. 1 filter or equivalent and a wide-bore funnel to ensure rapid flow. Leave the dish in the oven during the dipping process in order to keep the solution warm. Remove bubbles on top of the solution by touching them with a Kimwipe®.

D. Using a wire handle, immerse each slide rack in subbing solution for 3 min. Be careful not to allow bubbles to attach to the surface of the slides. To prevent this, immerse the rack slowly at a downward angle so that the frosted ends enter the solution last. Remove them in the reverse orientation, allowing 10 s for the solution to run toward the frosted end and begin to drip from the bottom of the rack before laying the rack on the foil. Thus, any excess solution will be accumulated over the frosted end of the slide.

E. Drain and separate the slides so that they do not stick to each other as they dry. To separate them, run an index finger or back end of a forceps across the top edges of the slides in the rack several times. This will also ensure proper draining of the excess solution onto the aluminum foil. Dry the slides overnight in a 37°C oven, and store them in boxes at 4°C away from dust.

7. Quick procedure

A. Cut 40 μm thick vibratome sections of selected brain regions, and float them in Tris-buffered saline (TBS).

B. Inactivate endogenous peroxidase activity in the tissues with methanol/hydrogen peroxide.

C–F. Wash sections in TBS. Microwave irradiate free-floating sections in citrate buffer for 5 min at 95°C. Cool to room temperature.

G–I. Wash in TBS, equilibrate with antibody diluting buffer (DB), and block non-specific protein binding sites with 20% normal goat serum in TBS for 1 h.

J. Incubate sections in working dilutions of primary antibodies in DB overnight at 4°C.

K. Wash in DB, and incubate in secondary antibodies for 1 h at room temperature.

L. Wash in DB and apply avidin-biotin-peroxidase complex prepared in TBS for 1 h.

M. Wash in TBS and develop with diaminobenzidine (DAB) using the DAB Substrate Kit for Peroxidase. Transfer sections to deionized water to stop DAB reaction.

N. If only a single label is required, skip to step O. If a double label is needed, wash sections in TBS and treat them with 50 mM Tris, pH 7.6, containing 10% dimethylformamide for 30 min. Then repeat steps I–M, using a VIP or SG Substrate Kit for Peroxidase as the chromogen for the second primary antibody.

O. Pick up sections on gelatin-subbed glass slides, and air-dry.

P. Identify and label the sections. Counterstain for Nissl substance or amyloid as needed.

8. Essential literature references

Original papers: Refs. [4–6,8,12–15,21,24,42,45–48,50,54,59].

Reviews: Refs. [10,16,25,30,33,53,55,56].

Books: Refs. [26,29,41].

References

[1] D.J. Barlow, M.S. Edwards, J.M. Thornton, Continuous and discontinuous protein antigenic determinants, Nature 322 (1986) 747–748.

[2] H. Battifora, M. Kopinski, The influence of protease digestion and duration of fixation on the immunostaining of keratins. A comparison of formalin and ethanol fixation, J. Histochem. Cytochem. 34 (1986) 1095–1100.

[3] S. Cammarata, G. Mancardi, M. Tabaton, Formic acid treatment exposes hidden neurofilament and tau epitopes in abnormal cytoskeletal filaments from patients with progressive supranuclear palsy and Alzheimer's disease, Neurosci. Lett. 115 (1990) 351–355.

[4] A.M. Cataldo, P.A. Paskevich, E. Kominami, R.A. Nixon, Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease, Proc. Natl. Acad. Sci. USA 88 (1991) 10998–11002.

[5] A.M. Cataldo, C.Y. Thayer, E.D. Bird, T.R. Wheelock, R.A. Nixon,

Lysosomal proteinase antigens are prominently localized within senile plaques of Alzheimer's disease: evidence for a neuronal origin. Brain Res. 513 (1990) 181–192.
[6] G. Cattoretti, M.H.G. Becker, G. Key, M. Duchrow, C. Schlüter, J. Galle, J. Gerdes, Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J. Pathol. 168 (1992) 357–363.
[7] G. Cattoretti, F. Doglioni, F. Fusilli, O. Zamboni, Microwave oven irradiation vs. trypsin digestion for antigen unmasking in fixed, paraffin-embedded material. Histochem. J. 24 (1992) 594.
[8] G. Cattoretti, S. Pileri, C. Parravicini, M.H.G. Becker, S. Poggi, C. Bifulco, G. Key, L. D'Amato, E. Sabattini, E. Feudale, F. Reynolds, J. Gerdes, F. Rilke, Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J. Pathol. 171 (1993) 83–98.
[9] A. Chakrabarty, L.R. Bridges, S. Gray, Cyclin D1 in astrocytic tumours: an immunohistochemical study. Neuropathol. Appl. Neurobiol. 22 (1996) 311–316.
[10] R.W. Dapson, Fixation for the 1990s: a review of needs and accomplishments. Biotechnol. Histochem. 68 (1993) 75–82.
[11] K.A. Dill, D. Shortle, Denatured states of proteins. Annu. Rev. Biochem. 60 (1991) 795–825.
[12] P. Evers, H.B.M. Uylings, Microwave-stimulated antigen retrieval is pH and temperature dependent. J. Histochem. Cytochem. 42 (1994) 1555–1563.
[13] P. Evers, H.B.M. Uylings, Effects of microwave pretreatment on immunocytochemical staining of vibratome sections and tissue blocks of human cerebral cortex stored in formaldehyde fixative for long periods. J. Neurosci. Methods 53 (1994) 163–172.
[14] P. Evers, H.B.M. Uylings, An optimal antigen retrieval method suitable for different antibodies on human brain tissue stored for several years in formaldehyde fixative. J. Neurosci. Methods 72 (1997) 197–207.
[15] H.K.P. Feirabend, S. Ploeger, Microwave applications in classical staining methods in formalin-fixed human brain tissue: a comparison between heating with microwaves and conventional ovens. Eur. J. Morphol. 29 (1991) 183–197.
[16] C.H. Fox, F.B. Johnson, J. Whiting, P.P. Roller, Formaldehyde fixation. J. Histochem. Cytochem. 33 (1985) 845–853.
[17] H. Fraenkel-Conrat, B.A. Brandon, H.S. Olcott, The reaction of formaldehyde with proteins IV. Participation of indole groups. Gramicidin, J. Biol. Chem. 168 (1947) 99–118.
[18] H. Fraenkel-Conrat, H.S. Olcott, Reaction of formaldehyde with proteins V. Cross-linking between amino and primary amide or guanidyl groups. J. Am. Chem. Soc. 70 (1948) 2673–2684.
[19] H. Fraenkel-Conrat, H.S. Olcott, Reaction of formaldehyde with proteins VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. J. Biol. Chem. 174 (1948) 827–843.
[20] D. French, J.T. Edsall, The reaction of formaldehyde with amino acids and proteins. Adv. Protein Chem. 2 (1945) 277–335.
[21] R. Gutiérrez, C. Bouras, P.R. Hof, P.G. Vallet, An improved thioflavine S method for staining neurofibrillary tangles and senile plaques in Alzheimer's disease. Experientia 48 (1992) 8–10.
[22] J.W. Harkin, S.H. Feairheller, Chemistry of the crosslinking of collagen during tanning, in: M. Friedman (Ed.), Protein Crosslinking. Symposium on Protein Crosslinking held in San Francisco, Plenum Press, New York, 1977, p. 425.
[23] H.M. Hazelhag, L.J. Van den Broek, E.B. Van Dunné, G.J. Offerhaus, G.J. Fleuren, P.C. Hogendoorn, Immunostaining of chain-specific keratins on formalin-fixed, paraffin-embedded tissues: a comparison of various antigen retrieval systems using microwave heating and proteolytic pretreatments. J. Histochem. Cytochem. 43 (1995) 429–437.
[24] K.G. Helander, Kinetics of formaldehyde binding in tissue. Biotechnol. Histochem. 69 (1994) 177–179.
[25] D. Hopwood, Fixatives and fixation: a review. Histochem. J. 1 (1969) 323–360.
[26] Horobin, R.W., Histochemistry. Butterworths, London, 1982.
[27] D.P. Kelly, M.K. Dewar, R.B. Johns, W.L. Shaw, J.F. Yates, Cross-linking of amino acids by formaldehyde. Preparation of ^{13}C NMR spectra of model compounds, in: M. Friedman (Ed.), Protein Crosslinking. Symposium on Protein Crosslinking held in San Francisco, Plenum Press, New York, 1977, p. 641.
[28] T. Kitamoto, K. Ogomori, J. Tateishi, S.B. Prusiner, Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Lab. Invest. 57 (1987) 230–236.
[29] L.P. Kok, M.F. Boon, Microwave Cookbook for Microscopists. Art and Science of Visualization, 3rd ed., Coulomb Press, Leiden, 1992.
[30] G.R. Login, A.M. Dvorak, Methods of microwave fixation for microscopy. A review of research and clinical applications: 1970–1992. Prog. Histochem. Cytochem. 27 (1994) 1–127.
[31] E. Murani, Instructions to contributing authors. Eur. J. Morphol. 29 (1991) 181.
[32] E. Murani, M.E. Boon, P.J. Adrians, W.J. Rietveld, L.P. Kok, Microwave-cryostat technique for neuroanatomical studies. J. Neurosci. Methods 22 (1987) 97–101.
[33] E. Murani, R.W. Horobin, Overview of microwave applications in the neurosciences. J. Neurosci. Methods 55 (1994) 111–117.
[34] J.T. Mason, T.J. O'Leary, Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. J. Histochem. Cytochem. 39 (1991) 225–229.
[35] S. McQuaid, R. McConnell, J. McMahon, B. Herron, Microwave antigen retrieval for immunocytochemistry on formalin-fixed, paraffin-embedded post-mortem CNS tissue. J. Pathol. 176 (1995) 207–216.
[36] G. Miettu, H. Jr, R. Ravi, Patterns of MiB-1 staining of brain tumors: microwave-antigen retrieval as a tool in diagnostic pathology. Eur. J. Morphol. 33 (1995) 337–358.
[37] H. Monnese, P. Mehta, H. Bantifaza, Antigen retrieval by microwave irradiation in lead thiocyanate. Comparison with protease digestion retrieval. Appl. Immunohistochem. 1 (1993) 77–82.
[38] S. Munakata, J.B. Hendricks, Effect of fixation time and microwave oven heating time on retrieval of the Ki-67 antigen from paraffin-embedded tissue. J. Histochem. Cytochem. 41 (1993) 1241–1246.
[39] A.J. Norton, Microwave oven heating for antigen unmasking in routinely processed tissue sections. J. Pathol. 171 (1993) 79–80.
[40] A.J. Norton, S. Jordan, P. Yeomans, Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J. Pathol. 173 (1994) 371–379.
[41] A.G.E. Pearse, Histochemistry, Theoretical and Applied, Vol. 1, 4th ed., Churchill Livingstone, London, 1980.
[42] S. Ploeger, J.M. Guldemond, H.K.P. Feirabend, E. Murani, Acidification of human brains stored in fixatives. Eur. J. Morphol. 31 (1993) 286–290.
[43] I. Podkletnova, K. Alho, Ultrasound-amplified immunohistochemistry. J. Histochem. Cytochem. 41 (1993) 51–56.
[44] F.E. Sherriff, L.R. Bridges, S.M. Gentleman, S. Sivaloganathan, S. Wilson, Markers of axonal injury in post mortem human brain. Acta Neuropathol. 88 (1994) 433–439.
[45] F.E. Sherriff, L.R. Bridges, P. Jackson, Microwave antigen retrieval of β-amyloid precursor protein immunoreactivity. NeuroReport 5 (1994) 1085–1088.
[46] S.-R. Shi, B. Chaiwun, L. Young, R.J. Cote, C.R. Taylor, Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J. Histochem. Cytochem. 41 (1993) 1599–1604.
[47] S.-R. Shi, J. Ou, K.L. Kalra, T. Chen, R.J. Cote, C.R. Taylor, Antigen retrieval technique: a novel approach to immunohistochemistry on routinely processed tissue sections. Cell Vision 2 (1995) 6–22.
[48] S.-R. Shi, M.E. Key, K.L. Kalra, Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohis-

technical staining based on microwave oven heating of tissue sections. J. Histochem. Cytochem. 39 (1991) 741–748.
[49] R.-W. Shin, T. Iwaki, T. Kitamoto, J. Tateishi. Hydrated autoclave pretreatment enhances tau immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. Lab. Invest. 64 (1991) 693–702.
[50] R.A. Shiurba, K. Ishiguro, M. Takahashi, K. Sato, G.T. Spooner, M. Mereken, R. Yoshida, T.R. Wheelock, H. Yanagawa, K. Imahori, R.A. Nixon. Immunocytochemistry of tau phosphoserine 413 and tau protein kinase I in Alzheimer pathology. Brain Res. 737 (1996) 119–132.
[51] J.D. Sipe, T.F. Ignaczak, P.S. Pollock, G.G. Glenner. Amyloid fibril protein AA: purification and properties of the antigenically related serum component as determined by solid phase radioimmunoassay. J. Immunol. 116 (1976) 1151–1156.
[52] H. Stein, K. Gatter, N. Asbahr, D.Y. Mason. Use of freeze-dried paraffin-embedded sections for immunohistologic staining with monoclonal antibodies. Lab. Invest. 52 (1985) 676–683.
[53] A.J.H. Suurmeijer. Optimizing immunohistochemistry in diagnostic tumor pathology with antigen retrieval. Eur. J. Morphol. 32 (1994) 325–334.
[54] A.J.H. Suurmeijer, M.E. Boon. Optimizing keratin and vimentin retrieval in formalin-fixed, paraffin-embedded tissue with the use of heat and metal salts. Appl. Immunohistochem. 1 (1993) 143–148.
[55] A.J.H. Suurmeijer, M.E. Boon. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. Eur. J. Morphol. 31 (1993) 144–150.
[56] P.E. Swanson. Microwave antigen retrieval in citrate buffer. Lab. Med. 25 (1994) 520–522.
[57] P.A. Takes, J. Kohrs, R. Krug, S. Kewley. Microwave technology in immunohistochemistry: application to avidin-biotin staining of diverse antigens. J. Histotechnol. 12 (1989) 95–98.
[58] C.R. Taylor, S.-R. Shi, C. Chen, L. Young, C. Yang, R.J. Cote. Comparative study of antigen retrieval heating methods: microwave, microwave and pressure cooker, autoclave, and steamer. Biotech. Histochem. 71 (1996) 263–270.
[59] P. Vandesande, K. Dierickx. Identification of the vasopressin producing and of the oxytocin producing neurons in the hypothalamic magnocellular neurosecretory system of the rat. Cell Tissue Res. 164 (1975) 153–162.
[60] R.K. Wolf, R. Busick, R. Schmidt-Kastner, P.K. Schmidt-Kastner, T. Pietsch, O.D. Wiestler, I. Blümcke. NeuN: a useful neuronal marker for diagnostic histopathology. J. Histochem. Cytochem. 44 (1996) 1167–1171.

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Protocol

Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections

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Abstract

Formalin fixation, the chemical process in which formaldehyde binds to cells and tissues, is widely used to preserve human brain specimens from autolytic decomposition [10, 16, 25, 41]. Ultrastructure of cellular and mitochondrial membranes is markedly altered by vesiculation [16], but this does not interfere with diagnostic evaluation of neurohistology by light microscopy. Serious difficulties are encountered, however, when immunocytochemical staining is attempted. Antigens that are immunoreactive in unfixed frozen sections and protein extracts appear to be concealed or destroyed in formalin-fixed tissues.

In dilute aqueous solution, formaldehyde is in equilibrium with methylene glycol and its polymeric hydrates, the balance by far in favor of methylene glycol [16, 41]. Carbonylic formaldehyde is a reactive electrophilic species well known for crosslinking functional groups in tissue proteins, nucleic acids, and polysaccharides [16, 17, 18, 19, 20, 22, 27]. Some of its methylene crosslinks are readily hydrolyzed. Others are stable and irreversible. During immunostaining reactions, intra- and inter-molecular links between macromolecules limit antibody permeation of tissue sections [16], alter protein secondary structure [26, 34], and reduce accessibility of antigenic determinants [10]. Accordingly, immunoreactivity is diminished for many antigens. Tissues are rapidly penetrated by methylene glycol, but formaldehyde binding to cellular constituents is relatively slow, increasing progressively until equilibrium is reached [16, 24]. In addition, prolonged storage in formalin may result in acidification of human brain specimens [42]. Low pH favors dissociation of methylene

glycol into formaldehyde [16], further reducing both classical staining and antigen detectability [14, 42].

Various procedures have been devised to counter the antigen masking effects of formaldehyde. Examples include pretreatment of tissue sections with proteases [2, 7, 23], formic acid [3, 28], or ultrasound [43]. Recently, heating of mounted sections in ionic salt solution by microwave energy was found to restore many antigens [6, 37, 44, 45, 46, 47, 48, 54, 55, 56]. Theory and practice of microwave antigen retrieval are covered extensively in the handbook *Microwave Cookbook for Microscopists* [29]. A concise overview of microwave methods in the neurosciences has been published [33], and clinical applications have been reviewed [30]. In this context, it should be noted that fresh tissues may be stabilized for immunocytochemistry by reversible, non-chemical binding processes such as cryosectioning after microwave treatment [32] and freeze-drying [52]. Thus, it may be possible to enhance immunostaining for some antigens by microwave irradiation of unfixed as well as fixed specimens.

Parameters to be optimized for microwave retrieval of specific antigens include temperature, irradiation time, tissue buffer composition, salt concentration, and pH [12, 47, 53]. Temperature, irradiation time, and pH are key variables [12, 13]. With this in mind, an optimal method was developed for retrieval of a wide variety of antigens in human brain tissues [14]. Typical microwave protocols employ elevated temperatures that may reach 100° C, where denaturation causes irreversible uncoiling and disruption of protein secondary and tertiary structures [11]. Under these conditions, stable covalent bonds securing methylene crosslinks between polypeptides remain intact [20], but more reactive links formed by Schiff bases may be hydrolyzed [45]. Resultant conformational changes presumably expose buried loops of continuous amino acids and protruding regions [1], increasing accessibility of their epitopes [51].

Protein denaturation seems to be a reasonable explanation for the effects of microwaves on antigen retrieval. This idea is supported by the observation that denaturing solutions such as 6 M urea increase immunoreactivity of some antigens [8]. Still, the molecular basis of these effects remains unresolved, in part due to the complex chemistry of formaldehyde reactions with tissue constituents [41]. Indeed, some methylene bridges between similar groups such as NH_2 and NH may be hydrolyzed by washing fixed tissues in distilled water at ambient temperature for several weeks [24, 41]. Moreover, denaturation by conventional heating enhances antigenicity as well as classical neuroanatomical staining of formalin-fixed tissue [15, 39, 40, 49]. When such externally heated specimens are immunostained and viewed by light microscopy, the results are almost indistinguishable from those obtained by microwave irradiation [58]. Nevertheless, the current widespread use of microwave methods in clinical and basic science laboratories likely results from the speed, convenience, and reproducibility of the results.

Loss of immunoreactivity for many antigens likewise may occur when tissues are dehydrated in alcohol before they are embedded in paraffin. Exposure to alcohol causes antigenic denaturation, but clearing in xylene and heating in liquid paraffin do not [52]. While there are numerous reports of microwave procedures for formalin-fixed, paraffin-embedded tissues [6, 8, 9, 35, 36, 38, 44, 45, 48, 57, 60], alternative methods that would further extend the range of retrievable epitopes have received less attention. One simple approach may be to irradiate free-floating sections with microwaves. A previous study found that heating vibratome sections in solution with microwaves resulted in severe wrinkling of the tissues. This problem was avoided by irradiation of tissue slices in buffer prior to sectioning [13]. Another strategy is presented here for neuropathological studies of human brain [50]. Vibratome sections in isotonic, mildly acidic citrate buffer [6, 55] are heated to the boiling point with microwaves. After brief denaturation at 100°C, the sections are simmered for 5 min. To

remove wrinkles, sections are incubated subsequently in Tris-buffered saline containing serum proteins and non-ionic detergent. The method also is suitable for single- and double-labeling studies of neural antigens in formalin-fixed tissues from experimental animals.

Author Keywords: Immunocytochemistry; Microwave antigen retrieval; Neuropathology; Tau protein kinase I/glycogen synthase kinase-3β

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